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PATENT  
09/051670

TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE (DO/US)—  
ENTRY INTO THE U.S. NATIONAL STAGE UNDER CHAPTER I

PCT/JP97/02859	19 August 1997	19 August 1996
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
NOVEL DNA AND PROCESS FOR PREPARING PROTEIN USING THE DNA		
TITLE OF INVENTION		
NAKAGAWA, Nobuaki; YASUDA, Hisataka		
APPLICANT(S)		

Box PCT  
Assistant Commissioner for Patents  
Washington D.C. 20231

ATTENTION: DO/US

NOTE: The completion of those filing requirements which can be made at a time later than 20 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 USC 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase. See 37 CFR 1.491 which states: "An international application enters the national stage when the applicant has filed the documents and fees required by 35 USC 371(c) within the periods set forth in § 1.494 and § 1.495."

**WARNING:** Where the items are those which can be submitted to complete the entry of the international application into the national phase subsequent to 20 months from the priority date the application is still considered to be in the international stage and if mailing procedures are utilized to obtain a date the express mail procedure of 37 CFR 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing - 37 CFR 1.8 (2) (xi)).

NOTE: Documents and fees must be clearly identified as a submission to enter the national stage under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 CFR 1.494(f).

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date April 16, 1998 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EM400954374US addressed to the Assistant Commissioner for Patents, Washington, D. C. 20231.

Paula M. Swirka

(type or print name of person mailing paper)



(Signature of person mailing paper)

NOTE: Each paper or fee referred to as enclosed herein has the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 CFR 1.16(b).

**WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 CFR 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

(Transmittal Letter to the United States Designated Office (DO/US)—Entry into National Stage Under 35 USC 371 [13-6]—page 1 of 6)

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1. Applicant herewith submits to the United States Designated Office (DO/US) the following items under 35 U.S.C. 371:

- a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).
- b. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 CFR 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
<input type="checkbox"/> *	TOTAL CLAIMS	4 —20=	0	× \$ 22.00=	\$
	INDEPENDENT CLAIMS	3 —3=	0	× \$ 82.00=	
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + \$270.00				
BASIC FEE**	The international search fee, as set forth in § 1.445(a)(2) to be paid to the US PTO acting as an international Searching Authority: <input type="checkbox"/> has been paid (37 CFR 1.492(a)(2)) ..... \$790.00 <input type="checkbox"/> has not been paid (37 CFR 1.492(a)(3)) ..... \$1070.00 <input checked="" type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) ..... \$930.00				930.00
	Total of above Calculations =				930.00
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 CFR 1.9, 1.27, 1.28)				—
	Subtotal				930.00
	Total National Fee \$				930.00
	Fee for recording the enclosed assignment document \$40.00 (37 CFR 1.21(h)). (See Item 10 below). See attached "ASSIGNMENT COVER SHEET (37 CFR 3.34)".				
TOTAL	Total Fees enclosed \$				930.00

\* See attached Preliminary Amendment Reducing the Number of Claims.

**\*\*WARNING:** "To avoid abandonment of the application, the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 20 months from the priority date: \* \* \* (2) the basic national fee (see § 1.492(a)). The 20-month time limit may not be extended " 37 CFR § 1.494(b).

(Transmittal Letter to the United States Designated Office (DO/US)—Entry into National Stage Under 35 USC 371 [13-6]—page 2 of 6)

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- i. ☒ A check in the amount of \$ 930.00 to cover the above fees is enclosed.
- ii. ☐ Please charge Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_. A duplicate copy of this sheet is enclosed.

**WARNING:** If the translation of the international application, oath or declaration and national fee have not been submitted by the applicant within twenty (20) months from the priority date, the applicant will be so notified and given a period of time within which to file the translation and/or oath or declaration in order to prevent abandonment. The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than twenty (20) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than twenty (20) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 will apply. 37 CFR § 1.494(c) and Notice of January 7, 1993, 1147 O.G. 29 to 40, at 35.

3. A copy of the International application as filed [35 U.S.C. 371(c)(2)]:
- a. ☐ is transmitted herewith.
- b. ☐ is not required as the application was filed with the United States Receiving Office.
- c. ☒ has been transmitted
- i. ☒ by the International Bureau. Date of mailing of the application (from form PCT/IB/308): 26 February 1998
- ii. ☐ by applicant on (date) \_\_\_\_\_

NOTE: Section 1.494(b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 20 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies the applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage and applicant has received notice from the International Bureau, applicant need only pay the basic national fee by 20 months from the priority date." Notice of January 7, 1993, 1147 O.G. 29 to 40, at 35.

4. ☒ A translation of the International application into the English language [35 U.S.C. 371(c)(2)]:
- a. ☒ is transmitted herewith.
- b. ☐ is not required as the application was filed in English.
- c. ☐ was previously transmitted by applicant on (date) \_\_\_\_\_
5. ☒ Amendments to the claims of the International application under PCT Article 19 [35 U.S.C. 371(c)(3)]:

NOTE: The Notice of January 7, 1993 indicates that 37 CFR § 1.494(d) was "amended to clarify the existing practice that PCT Article 19 Amendments must be submitted by 20 months from the priority date, which time may not be extended." This Notice further advises: "Of course, the failure to do so does not result in loss of the subject matter of PCT Article 19 amendments. The applicant may submit that subject matter in a preliminary amendment filed under Section 1.121. In many cases, filing an amendment under Section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 35. See item 11(c) below.

- a. ☐ are transmitted herewith.

(Transmittal Letter to the United States Designated Office (DO/US)—Entry into National Stage Under 35 USC 371 [13-6]—page 3 of 6)

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- b. ☐ have been transmitted
- i. ☐ by the International Bureau. Date of mailing of the amendment (from form PCT/IB/308): \_\_\_\_\_.
- ii. ☐ by applicant on (date) \_\_\_\_\_.
- c. ☒ have not been transmitted as
- i. ☐ no notification has been received that the International Search Authority has received the Search Copy.
- ii. ☐ the Search Copy was received by the International Searching Authority but the Search Report has not yet been issued. Date of receipt of Search Copy (from form PCT/ISA/202): \_\_\_\_\_.
- iii. ☒ applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210.): Sept. 29, 1997
- iv. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☒ A translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]:

- a. ☐ is transmitted herewith.
- b. ☐ is not required as the amendments were made in the English language.
- c. ☒ has not been transmitted for reasons indicated at point 5.c. above.

7. ☒ An oath or declaration of the inventor [35 U.S.C. 371(c)(4)] complying with 35 U.S.C. 115

- a. ☐ was previously submitted by applicant on (date) \_\_\_\_\_.
- b. ☐ is submitted herewith and such oath or declaration
- i. ☐ is attached to the application.
- ii. ☐ identifies the application and any amendments under PCT Article 19 which were transmitted as stated in points 3.b. or c. and 5.b; and states that they were reviewed by the inventor as required by 37 CFR 1.70.
- iii. ☒ will follow.

II. Other document(s) or information included:

8. ☒ An international Search Report or Declaration under PCT Article 17(2)(a):

- a. ☐ is transmitted herewith.
- b. ☒ has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): 26 February 1998
- c. ☐ is not required as the application was searched by the United States International Searching Authority.

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- d. ☐ will be transmitted promptly upon request.
- e. ☐ has been submitted by applicant on (date) \_\_\_\_\_.
- f. ☐ is not transmitted as the international search has not yet issued.
9. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98:
- a. ☐ is transmitted herewith.
- Also transmitted herewith is
- ☐ Form PTO—1449
- ☐ Copies of citations listed
- b. ☒ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
- c. ☐ was previously submitted by applicant on (date) \_\_\_\_\_.
10. ☐ An assignment document is transmitted herewith for recording. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO—1595 is also attached.
- ☐ Please mail the recorded assignment document to:
- i. ☐ the person whose signature and address appears below.
- ii. ☐ the following:
11. ☒ Additional documents
- a. ☐ Copy of request (PCT/RO/101)
- b. ☒ International Publication No. W098/07840
- i. ☐ Specification, claims and drawing
- ii. ☒ Front page only
- c. ☐ Preliminary amendment (37 CFR § 1.121)
- d. ☒ Other : Certificate of Biological Deposit  
Computer Readable Copy of Sequence Listing (Disk)
12. ☒ The above checked items are being transmitted
- a. ☐ before the 18th month publication.
- b. ☒ after publication and the article 20 communication but before 20 months from the priority date.
- c. ☐ after 20 months (revival).
- NOTE: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements are submitted after 20 months.
13. ☐ Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on \_\_\_\_\_ (date) namely:

# AUTHORIZATION TO CHARGE ADDITIONAL FEES

**WARNING:** Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.

- ☒ The Commissioner is hereby authorized to charge the following additional fees which may be required by this paper and during the entire pendency of this application to Account No. 20-0531.

☒ 37 CFR 1.492(a)(1), (2), (3), and (4) (filing fees)

**WARNING:** Because failure to pay the national fee within 20 months without extension (37 CFR § 1.494(b)(2)), results in abandonment of the application, it would be best to always check the above box.

☒ 37 CFR 1.492(b), (c), and (d) (presentation of extra claims)

**NOTE:** Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☐ 37 CFR 1.17 (application processing fees)


**WARNING:** While 37 CFR 1.17(a), (b), (c) and (d) deal with extensions of time under § 1.136(a) this authorization should be made only with the knowledge that: "Submission of the appropriate extension fee under 37 CFR 1.136(a) is to no avail unless a request or petition for extension is filed" (Emphasis added). Notice of November 5, 1985 (1060 O.G. 27).

☐ CFR 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 CFR 1.311(b).

**NOTE:** Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 CFR 1.311(b).

**NOTE:** 37 CFR 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee". From the wording of 37 CFR 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

☐ 37 CFR 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 20 months after the priority date.

  
Signature of attorney  
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### DESCRIPTION

NOVEL DNA AND PROCESS FOR PREPARING PROTEIN USING THE DNA

### FIELD OF TECHNOLOGY

The present invention relates to a novel DNA and a process for preparing a protein which possesses an activity to inhibit osteoclast differentiation and/or maturation (hereinafter called osteoclastogenesis-inhibitory activity) by a genetic engineering technique using the DNA. More particularly, the present invention relates to a genomic DNA encoding a protein OCIF which possesses an osteoclastogenesis-inhibitory activity and a process for preparing said protein by a genetic engineering technique using the genomic DNA.

### BACKGROUND OF THE INVENTION

Human bones are constantly repeating a process of resorption and formation. Osteoblasts controlling formation of bones and osteoclasts controlling resorption of bones take major roles in this process. Osteoporosis is a typical disease caused by abnormal metabolism of bones. This disease is caused when bone resorption by osteoclasts exceeds bone formation by osteoblasts. Although the mechanism of this disease is still to be elucidated completely, the disease causes the bones to ache, makes the bones fragile, and may results in fracturing of the bones. As the population of the aged increases, this disease results in an increase in bedridden aged people which becomes a social problem. Urgent development of a therapeutic agent for this disease is strongly desired. Disease due to a

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decrease in bone mass is expected to be treated by controlling bone resorption, accelerating bone formation, or improving balance between bone resorption and formation.

Osteogenesis is expected to increase by accelerating proliferation, differentiation, or activation of the cells controlling bone formation, or by controlling proliferation, differentiation, or activation of the cells involved in bone resorption. In recent years, strong interest has been directed to physiologically active proteins (cytokines) exhibiting such activities as described above, and energetic research is ongoing on this subject. The cytokines which have been reported to accelerate proliferation or differentiation of osteoblasts include the proteins of fibroblast growth factor family (FGF: Rodan S. B. et al., Endocrinology vol. 121, p 1917, 1987), insulin-like growth factor I (IGF-I: Hock J. M. et al., Endocrinology vol. 122, p 254, 1988), insulin growth factor II (IGF-II: McCarthy T. et al., Endocrinology vol. 124, p 301, 1989), Activin A (Centrella M. et al., Mol. Cell. Biol., vol. 11, p 250, 1991), transforming growth factor- $\beta$ , (Noda M., The Bone, vol. 2, p 29, 1988), Vasculotropin (Varonique M. et al., Biochem. Biophys. Res. Commun., vol. 199, p 380, 1994), and the protein of heterotopic bone formation factor family (bone morphogenic protein; BMP: BMP-2; Yanaguchi A. et al., J. Cell Biol. vol. 113, p 682, 1991, OP-1; Sampath T. K. et al., J. Biol. Chem. vol. 267, p 20532, 1992, and Knutsen R. et al., Biochem. Biophys. Res. Commun. vol. 194, P 1352, 1993).

On the other hand, as the cytokines which suppress



differentiation and/or maturation of osteoclasts, transforming growth factor- $\beta$  (Chenu C, et. al., Proc. Natl. Acad. Sci. USA, vol. 85, p 5683, 1988), interleukin-4 (Kasano K. et al., Bone-Miner., vol. 21, p 179, 1993), and the like have been reported. Further, as the cytokines which suppress bone resorption by osteoclast, calcitonin (Bone-Miner., vol. 17, p 347, 1992 ), macrophage colony stimulating factor (Hattersley G. et al., J. Cell. Physiol. vol. 137, p 199. 1988), interleukin-4 (Watanabe, K. et al., Biochem. Biophys. Res. Commun. vol. 172. P 1035, 1990), and interferon- $\gamma$  (Gowen M. et al., J. Bone Miner. Res., vol. 1, p 46.9, 1986) have been reported.

These cytokines are expected to be used as agents for treating diseases accompanying bone loss by accelerating bone formation or suppressing of bone resorption. Clinical tests are being undertaken to verify the effect of improving bone metabolism of some cytokines such as insulin-like growth factor-I and the heterotopic bone formation factor family. In addition, calcitonin is already commercially available as a therapeutic agent for osteoporosis and a pain relief agent. At present, drugs for clinically treating bone diseases or shortening the period of treatment of bone diseases include activated vitamin D<sub>3</sub>, calcitonin and its derivatives, and hormone preparations such as estradiol agent, ipriflavon or calcium preparations. These agents are not necessarily satisfactory in terms of the efficacy and therapeutic results. Development of a novel therapeutic agent which can be used in

place of these agents is strongly desired.

In view of this situation, the present inventors have undertaken extensive studies. As a result, the present inventors had found protein OCIF exhibiting an osteoclastogenesis-inhibitory activity in a culture broth of human embryonic lung fibroblast IMR-90 (ATCC Deposition No. CCL186), and filed a patent application (PCT/JP96/00374). The present inventors have conducted further studies relating to the origin of this protein OCIF exhibiting the osteoclastogenesis-inhibitory activity. The studies have matured into determination of the sequence of a genomic DNA encoding the human origin OCIF. Accordingly, an object of the present invention is to provide a genomic DNA encoding protein OCIF exhibiting osteoclastogenesis-inhibitory activity and a process for preparing this protein by a genetic engineering technique using the genomic DNA.

#### DISCLOSURE OF THE INVENTION

Specifically, the present invention relates to a genomic DNA encoding protein OCIF exhibiting osteoclastogenesis-inhibitory activity and a process for preparing this protein by a genetic engineering technique using the genomic DNA. The DNA of the present invention includes the nucleotide sequences No. 1 and No. 2 in the Sequence Table attached hereto.

Moreover, the present invention relates to a process for preparing a protein, comprising inserting a DNA including the nucleotide sequences of the sequences No. 1 and No. 2 in the Sequence Table into an expression vector, producing a vector

capable of expressing a protein having the following physicochemical characteristics and exhibiting the activity of inhibiting differentiation and/or maturation of osteoclasts, and producing this protein by a genetic engineering technique,

(a) molecular weight (SDS-PAGE):

(i) Under reducing conditions: about 60 kD,

(ii) Under non-reducing conditions: about 60 kD and about 120 kD;

(b) amino acid sequence:

includes an amino acid sequence of the Sequence ID No. 3 of the Sequence Table,

(c) affinity:

exhibits affinity to a cation exchanger and heparin, and

(d) thermal stability:

(i) the osteoclast differentiation and/or maturation inhibitory activity is reduced when treated with heat at 70°C for 10 minutes or at 56°C for 30 minutes,

(ii) the osteoclast differentiation and/or maturation inhibitory activity is lost when treated with heat at 90°C for 10 minutes.

The protein obtained by expressing the gene of the present invention exhibits an osteoclastogenesis-inhibitory activity. This protein is effective as an agent for the treatment and improvement of diseases involving decrease in the amount of bone such as osteoporosis, diseases relating to bone metabolism abnormality such as rheumatism, degenerative joint disease, or multiple myeloma, and is useful as an antigen to establish an

immunological diagnosis of such diseases.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a result of Western Blotting analysis of the protein obtained by causing genomic DNA of the present invention to express a protein in Example 4 (iii), wherein lane 1 indicates a marker, lane 2 indicates the culture broth of COS7 cells in which a vector pWESR $\alpha$ OCIF (Example 4 (iii)) has been transfected, and lane 3 is the culture broth of COS7 cell in which a vector pWESR $\alpha$  (control) has been transfected.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The genomic DNA encoding the protein OCIF which exhibits osteoclastogenesis-inhibitory activity in the present invention can be obtained by preparing a cosmid library using a human placenta genomic DNA and a cosmid vector and by screening this library using DNA fragments which are prepared based on the OCIF cDNA as a probe. The thus-obtained genomic DNA is inserted into a suitable expression vector to prepare an OCIF expression cosmid. A recombinant type OCIF can be obtained by transfecting the genomic DNA into a host organism such as various types of cells or microorganism strains and causing the DNA to express a protein by a conventional method. The resultant protein exhibiting osteoclastogenesis-inhibitory activity (an osteoclastogenesis-inhibitory factor) is useful as an agent for the treatment and improvement of diseases involving a decrease in bone mass such as osteoporosis and other diseases relating to bone metabolism abnormality and also as an antigen to prepare antibodies for establishing immunological diagnosis of such

diseases. The protein of the present invention can be prepared as a drug composition for oral or non-oral administration. Specifically, the drug composition of the present invention containing the protein which is an osteoclastogenesis-inhibitory factor as an active ingredient can be safely administered to humans and animals. As the form of drug composition, a composition for injection, composition for intravenous drip, suppository, nasal agent, sublingual agent, percutaneous absorption agent, and the like are given. In the case of the composition for injection, such a composition is a mixture of a pharmacologically effective amount of osteoclastogenesis-inhibitory factor of the present invention and a pharmaceutically acceptable carrier. The composition may further comprise amino acids, saccharides, cellulose derivatives, and other excipients and/or activation agents, including other organic compounds and inorganic compounds which are commonly added to a composition for injection. When an injection preparation is prepared using the osteoclastogenesis-inhibitory factor of the present invention and these excipients and activation agents, a pH adjuster, buffering agent, stabilizer, solubilizing agent, and the like may be added if necessary to prepare various types of injection agents.

The present invention will now be described in more detail by way of examples which are given for the purpose of illustration and not intended to be limiting of the present invention.

Example 1

<Preparation of a cosmid library>

A cosmid library was prepared using human placenta genomic DNA (Clonotech; Cat. No. 6550-2) and pWE15 cosmid vector (Stratagene). The experiment was carried out following principally the protocol attached to the pWE15 cosmid vector kit of Stratagene Company, provided Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory (1989)) was referred to for common procedures for handling DNA, E. coli, and phage.

(i) Preparation of restrictive enzymolysate of human-genomic DNA

Human placenta genomic DNA dissolved in 750  $\mu$ l of a solution containing 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, and 100 mM NaCl was added to four 1.5 ml Eppendorf tubes (tube A, B, C, and D) in the amount of 100  $\mu$ g each. Restriction enzyme MboI was added to these tubes in the amounts of 0.2 unit for tube A, 0.4 unit for tube B, 0.6 unit for tube C, and 0.8 unit for tube D, and DNA was digested for 1 hour. Then, EDTA in the amount to make a 20 mM concentration was added to each tube to terminate the reaction, followed by extraction with phenol/chloroform (1:1). A two-fold amount of ethanol was added to the aqueous layer to precipitate DNA. DNA was collected by centrifugation, washed with 70% ethanol, and DNA in each tube was dissolved in 100  $\mu$ l of TE (10 mM HCl (pH 8.0) + 1 mM EDTA buffer solution, hereinafter called TE). DNA in four tubes was combined in one tube and incubated for 10 minutes at 68°C. After cooling to room

temperature, the mixture was overlayed onto a 10%-40 % linear sucrose gradient which was prepared in a buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1 mM NaCl in an centrifugal tube (38 ml). The tube was centrifuged at 26,000 rpm for 24 hours at 20°C using a rotor SRP28SA manufactured by Hitachi, Ltd. and 0.4 ml fractions of the sucrose gradient was collected using a fraction collector. A portion of each fraction was subjected to 0.4% agarose electrophoresis to confirm the size of DNA. Fractions containing DNA with a length of 30 kb (kilo base pair) to 40 kb were thus combined. The DNA solution was diluted with TE to make a sucrose concentration to 10% or less and 2.5-fold volumes of ethanol was added to precipitate DNA. DNA was dissolved in TE and stored at 4°C.

(ii) Preparation of cosmid vector

The pWE15 cosmid vector obtained from Stratagene Company was completely digested with restriction enzyme BamHI according to the protocol attached to the cosmid vector kit. DNA collected by ethanol precipitation was dissolved in TE to a concentration of 1 mg/ml. Phosphoric acid at the 5'-end of this DNA was removed using calf small intestine alkaline phosphatase, and DNA was collected by phenol extraction and ethanol precipitation. The DNA was dissolved in TE to a concentration of 1 mg/ml.

(iii) Ligation of genomic DNA to vector and in vitro packaging

1.5 micrograms of genomic DNA fractionated according to size and 3 µg of pWE15 cosmid vector which was digested with

restriction enzyme BamHI were ligated in 20  $\mu$ l of a reaction solution using Ready-To-Go T4DNA ligase of Pharmacia Company. The ligated DNA was packaged in vitro using Gigapack™ II packaging extract (Stratagene) according to the protocol. After the packaging reaction, a portion of the reaction mixture was diluted stepwise with an SM buffer solution and mixed with E. coli XL1-Blue MR (Stratagene) which was suspended in 10 mM  $MgCl_2$  to cause phage to infect, and plated onto LB agar plates containing 50  $\mu$ g/ml of ampicillin. The number of colonies produced was counted. The number of colonies per 1  $\mu$ l of packaging reaction was calculated based on this result.

(iv) Preparation of a cosmid library

The packaging reaction solution thus prepared was mixed with E. coli XL1-Blue MR and the mixture was plated onto agarose plates containing ampicillin so as to produce 50,000 colonies per agarose plate having a 15 cm of diameter. After incubating the plate overnight at 37°C, an LB culture medium was added in the amount of 3 ml per plate to suspend and collect colonies of E. coli. Each agarose plate was again washed with 3 ml of the LB culture medium and the washing was combined with the original suspension of E. coli. The E. coli collected from all agarose plates was placed in a centrifugal tube, glycerol was added to a concentration of 20%, and ampicillin was further added to make a final concentration of 50  $\mu$ g/ml. A portion of the E. coli suspension was removed and the remainder was stored at -80°C. The removed E. coli was diluted stepwise and plated onto an agar plates to count the number of colonies per 1 ml of



suspension.

## Example 2

### <Screening of cosmid library and purification of colony>

A nitrocellulose filter (Millipore) with a diameter of 14.2 cm was placed on each LB agarose plate with a diameter of 15 cm which contained 50 µg/ml of ampicillin. The cosmid library was plated onto the plates so as to produce 50,000 colonies of *E. coli* per plate, followed by incubation overnight at 37°C. *E. coli* on the nitrocellulose filter was transferred to another nitrocellulose filter according to a conventional method to obtain two replica filters. According to the protocol attached to the cosmid vector kit, cosmid DNA in the *E. coli* on the replica filters was denatured with an alkali, neutralized, and immobilized on the nitrocellulose filter using a Stratalinker (Stratagene). The filters were heated for two hours at 80°C in a vacuum oven. The nitrocellulose filters thus obtained were hybridized using two kinds of DNA produced, respectively, from 5'-end and 3'-end of human OCIF cDNA as probes. Namely, a plasmid was purified from *E. coli* pKB/OIF10 (deposited at The Ministry of International Trade and Industry, the Agency of Industrial Science and Technology, Biotechnology Laboratory, Deposition No. FERM BP-5267) containing OCIF cDNA. The plasmid containing OCIF cDNA was digested with restriction enzymes KpnI and EcoRI. Fragments thus obtained was separated using agarose gel electrophoresis. KpnI/EcoRI fragment with a length of 0.2 kb was purified using a QIAEX II gel extraction kit (Qiagen). This DNA was labeled with <sup>32</sup>p using the Megaprime DNA Labeling

System (Amasham) (5'-DNA probe). Apart from this, a BamHI/EcoRV fragment with a length of 0.2 kb which was produced from the above plasmid by digestion with restriction enzymes BamHI and EcoRV was purified and labeled with  $^{32}\text{p}$  (3'-DNA probe). One of the replica filters described above was hybridized with the 5'-DNA probe and the other with the 3'-DNA probe. Hybridization and washing of the filters were carried out according to the protocol attached to the cosmid vector kit. Autoradiography detected several positive signals with each probe. One colony which gave positive signals with both probe was identified. The colony on the agar plate, which corresponding to the signal on the autoradiogram was isolated and purified. A cosmid was prepared from the purified colony by a conventional method. This cosmid was named pWEOCIF. The size of human genomic DNA contained in this cosmid was about 38 kb.

### Example 3

#### <Determination of the nucleotide sequence of human OCIF genomic DNA>

##### (i) Subcloning of OCIF genomic DNA

Cosmid pWEOCIF was digested with restriction enzyme EcoRI. After the separation of the DNA fragments thus produced by electrophoresis using a 0.7% agarose gel, the DNA fragments were transferred to a nylon membrane (Hybond -N, Amasham) by the Southern blot technique and immobilized on the nylon membrane using Stratalinker (Stratagene). On the other hand, plasmid pBKOCIF was digested with restriction enzyme EcoRI and a 1.6

kb fragment containing human OCIF cDNA was isolated by agarose gel electrophoresis. The fragment was labeled with  $^{32}\text{P}$  using the Megaprime DNA labeling system (Amasham).

Hybridization of the nylon membranes described above with the  $^{32}\text{P}$ -labeled 1.6-kb OCIF cDNA was performed according to a conventional method detected that DNA fragments with a size of 6 kb, 4 kb, 3.6 kb, and 2.6 kb. These fragments hybridized with the human OCIF cDNA were isolated using agarose gel electrophoresis and individually subcloned into an EcoRI site of pBluescript II SK + vector (Strategene) by a conventional method. The resulting plasmids were respectively named pBSE 6, pBSE 4, pBSE 3.6, and PBSE 2.6.

(ii) Determination of the nucleotide sequence

The nucleotide sequence of human OCIF genomic DNA which was subcloned into the plasmid was determined using the ABI Dideoxy Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and the 373 Sequencing System (Applied Biosystems). The primer used for the determination of the nucleotide sequence was synthesized based on the nucleotide sequence of human OCIF cDNA (Sequence ID No. 4 in the Sequence Table). The nucleotide sequences thus determined are given as the Sequences No. 1 and No. 2 in the Sequence Table. The Sequence ID No. 1 includes the first exon of the OCIF gene and the Sequence ID No. 2 includes the second, third, fourth, and fifth exons. A stretch of about 17 kb is present between the first and second exons.

Example 4

<Production of recombinant OCIF using COS-7 cells>

(i) Preparation of OCIF genomic DNA expression cosmid

To express OCIF genomic DNA in animal cells, an expression unit of expression plasmid pcDL-SR $\alpha$ 296 (Molecular and Cellular Biology, vol. 8, P466-472, 1988) was inserted into cosmid vector pWE15 (Stratagene). First of all, the expression plasmid pcDL-SR $\alpha$ 296 was digested with a restriction enzyme Sal I to cut out expression unit with a length of about 1.7 kb which includes an SR $\alpha$ promotor, SV40 later splice signal, poly (A) addition signal, and so on. The digestion products were separated by agarose electrophoresis and the 1.7-kb fragment was purified using the QIAEX II gel extraction kit (Qiagen). On the other hand, cosmid vector pWE15 was digested with a restriction enzyme EcoRI and fragments were separated using agarose gel electrophoresis. pWE15 DNA of 8.2 kb long was purified using the QIAEX II gel extraction kit (Qiagen). The ends of these two DNA fragments were bluntled using a DNA blunting kit (Takara Shuzo), ligated using a DNA ligation kit (Takara Shuzo), and transferred into E. coli DH5 $\alpha$  (Gibco BRL). The resultant transformant was grown and the expression cosmid pWESR $\alpha$  containing an expression unit was purified using a Qiagen column (Qiagen).

The cosmid pWE OCIF containing the OCIF genomic DNA with a length of about 38 kb obtained in (i) above was digested with a restriction enzyme NotI to cut out the OCIF genomic DNA of about 38 kb. After separation by agarose gel electrophoresis, the DNA was purified using the QIAEX II gel extraction kit (Qiagen). On the other hand, the expression cosmid pWESR $\alpha$  was

digested with a restriction enzyme EcoRI and the digestion product was extracted with phenol and chloroform, ethanol-precipitated, and dissolved in TE.

pWESR $\alpha$  digested with a restriction enzyme EcoRI and an EcoRI-XmnI-NotI adapter (#1105, #1156 New England Biolaboratory Co.) were ligated using T4 DNA ligase (Takara Shuzo Co., Ltd.). After removal of the free adapter by agarose gel electrophoresis, the product was purified using QIAEX gel extraction kit (Qiagen). The OCIF genomic DNA with a length of about 37 kb which was derived from the digestion with restriction enzyme NotI and the pWESR $\alpha$  to which the adapter was attached were ligated using T4 DNA ligase (Takara Shuzo). The DNA was packaged in vitro using the Gigapack packaging extract (Stratagene) and infected with E. coli XL1-Blue MR (Stratagene). The resultant transformant was grown and the expression cosmid pWESR $\alpha$ OCIF which contained OCIF genomic DNA was inserted was purified using a Qiagen column (Qiagen). The OCIF expression cosmid pWESR $\alpha$ OCIF was ethanol-precipitated and dissolved in sterile distilled water and used in the following analysis.

(ii) Transient expression of OCIF genomic DNA and measurement of OCIF activity

A recombinant OCIF was expressed as described below using the OCIF expression cosmid pWESR $\alpha$ OCIF obtained in (i) above and its activity was measured. COS-7 ( $8 \times 10^5$  cells/well) cells (Riken Cell Bank, RCB0539) were planted in a 6-well plate using DMEM culture medium (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL). On the following day, the culture

medium was removed and cells were washed with serum-free DMEM culture medium. The OCIF expression cosmid pWESR $\alpha$ OCIF which had been diluted with OPTI-MEM culture medium (Gibco BRL) was mixed with lipopfectamine and the mixture was added to the cells in each well according to the attached protocol. The expression cosmid pWESR $\alpha$  was added to the cells in the same manner as a control. The amount of the cosmid DNA and Lipopfectamine was respectively 3  $\mu$ g and 12  $\mu$ l. After 24 hours, the culture medium was removed and 1.5 ml of fresh EX-CELL 301 culture medium (JRH Bioscience) was added to each well. The culture medium was recovered after 48 hours and used as a sample for the measurement of OCIF activity. The measurement of OCIF activity was carried out according to the method described by Kumegawa, M. et al. (Protein, Nucleic Acid, and Enzyme, Vol. 34, p 999 (1989)) and the method of TAKAHASHI, N. et al. (Endocrinology vol. 122, p 1373 (1988)). The osteoclast formation in the presence of activated vitamin D<sub>3</sub> from bone marrow cells isolated from mice aged about 17 days was evaluated by the induction of tartaric acid resistant acidic phosphatase activity. The inhibition of the acid phosphatase was measured and used as the activity of the protein which possesses osteoclastogenesis-inhibitory activity (OCIF). Namely, 100  $\mu$ l/well of a OCIF sample which was diluted with  $\alpha$ -MEM culture medium (Gibco BRL) containing  $2 \times 10^{-8}$  M activated vitamin D<sub>3</sub> and 10% fetal bovine serum was added to each well of a 96 well micro plate. Then,  $3 \times 10^5$  bone marrow cells isolated from mice (about 17-days old) suspended in 100  $\mu$ l of  $\alpha$ -MEM culture medium containing 10% fetal bovine serum

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were added to each well of the 96 well micro plate and cultured for a week at 37°C and 100% humidity under 5% CO<sub>2</sub> atmosphere. On days 3 and 5, 160 µl of the conditioned medium was removed from each well, and 160 µl of a sample which was diluted with α-MEM culture medium containing 1x10<sup>-8</sup> M activated vitamin D<sub>3</sub> and 10% fetal bovine serum was added. After 7 days from the start of culturing, the cells were washed with a phosphate buffered saline and fixed with a ethanol/acetone (1:1) solution for one minute at room temperature. The osteoclast formation was detected by staining the cells using an acidic phosphatase activity measurement kit (Acid Phosphatase, Leucocyte, Cat. No. 387-A, Sigma Company). A decrease in the number of cells positive to acidic phosphatase activity in the presence of tartaric acid was taken as the OCIF activity. The results are shown in Table 1, which indicates that the conditioned medium exhibits the similar activity to natural type OCIF obtained from the IMR-90 culture medium and recombinant OCIF produced by CHO cells.

TABLE 1

Activity of OCIF expressed by COS-7 cells in the conditioned medium

Dilution	1/10	1/20	1/40	1/80	1/160	1/320
OCIF genomic DNA introduced	++	++	++	++	+	-
Vector introduced	-	-	-	-	-	-
Untreated	-	-	-	-	-	-

"++" indicates an activity inhibiting 80% or more of osteoclast formation, "+" indicates an activity inhibiting 30-80% of osteoclast formation, and "-" indicates that no inhibition of osteoclast formation is observed.

(iii) Identification of the product by Western Blotting

A buffer solution (10  $\mu$ l) for SDS-PAGE (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20  $\mu$ g/ml bromophenol blue, pH 6.8) was added to 10  $\mu$ l of the sample for the measurement of OCIF activity prepared in (ii) above. After boiling for 3 minutes at 100°C, the mixture was subjected to 10% SDS polyacrylamide electrophoresis under non-reducing conditions. The proteins were transferred from the gel to a PVDF membrane (ProBlott, Perkin Elmer) using semi-dry blotting apparatus (Biorad). The membrane was blocked and incubated for 2 hours at 37°C together with a horseradish peroxidase-labeled anti-OCIF antibody obtained by labeling the previously obtained OCIF protein with horseradish peroxidase according to a conventional method. After washing, the protein which has bound the anti-OCIF antibody was detected using the ECL system (Amasham). As shown in Figure 1, two bands, one with a molecular weight of about 120 kilo dalton and the other 60 kilo dalton, were detected in



the supernatant obtained from the culture broth of COS-7 cells in which pWESR $\alpha$ OCIF was transfected. On the other hand, these two bands with a molecular weight of about 120 kilo dalton and 60 kilo dalton were not detected in the supernatant obtained from the culture broth of COS-7 cells in which pWESR $\alpha$ vector was transfected, confirming that the protein obtained was OCIF.

#### INDUSTRIAL APPLICABILITY

The present invention provides a genomic DNA encoding a protein OCIF which possesses an osteoclastogenesis-inhibitory activity and a process for preparing this protein by a genetic engineering technique using the genomic DNA. The protein obtained by expressing the gene of the present invention exhibits an osteoclastogenesis-inhibitory activity and is useful as an agent for the treatment and improvement of diseases involving a decrease in the amount of bone such as osteoporosis, other diseases resulting from bone metabolism abnormality such as rheumatism or degenerative joint disease, and multiple myeloma. The protein is further useful as an antigen to establish antibodies useful for an immunological diagnosis of such diseases.

#### NOTE ON MICROORGANISM

Depositing Organization:

The Ministry of International Trade and Industry, National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology

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852050" 029T5060

# TABLE OF SEQUENCES

Sequence number: 1

Length of sequence: 1316

Sequence Type: nucleic acid

Strandedness: double

Topology: linear

Molecular type: genomic DNA (human OCIF genomic DNA-1)

Sequence:

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-20

-15

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Ala Leu Val

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Sequence number: 2

Length of sequence: 9898

Sequence Type: nucleic acid

Strandedness: double

Topology: linear

Molecular type: genomic DNA (human OCIF genomic DNA-2)

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Phe Leu Asp Ile Ser Ile Lys Trp Thr Thr Gln Glu Thr Phe

-10

-5

1

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5

10

15

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GTCTACTTAT ATATCTGTAT CTATCTCTTG CTTTGTTC AAAGGTAAAC TATGTGTCTA 6275  
AATGTGGGCA AAAAATAACA CACTATTCCA AATTACTGTT CAAATTCCTT TAAGTCAGTG 6335  
ATAATTATTT GTTTTGACAT TAATCATGAA GTTCCCTGTG GGTACTAGGT AAACCTTTAA 6395  
TAGAATGTTA ATGTTTGTAT TCATTATAAG AATTTTTGGC TGTTACTTAT TTACAACAAT 6455  
ATTTCACTCT AATTAGACAT TTAATAAAT TTCTCTTGAA AACAATGCCC AAAAAAGAAC 6515  
ATTAGAAGAC ACGTAAGCTC AGTTGGTCTC TGCCACTAAG ACCAGCCAAC AGAAGCTTGA 6575  
TTTTATTCAA ACTTTGCATT TTAGCATATT TTATCTTGGA AAATTCAATT GTGTTGGTTT 6635  
TTTGTTTTTG TTTGTATTGA ATAGACTCTC AGAAATCCAA TTGTTGAGTA AATCTTCTGG 6695  
GTTTTCTAAC CTTTCTTTAG AT GTT ACC CTG TGT GAG GAG GCA TTC TTC AGG 6747

Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg

180

185

TTT GCT GTT CCT ACA AAG TTT ACG CCT AAC TGG CTT AGT GTC TTG GTA 6795  
Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val

190

195

200

GAC AAT TTG CCT GGC ACC AAA GTA AAC GCA GAG AGT GTA GAG AGG ATA 6843  
Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile

205

210

215

AAA CGG CAA CAC AGC TCA CAA GAA CAG ACT TTC CAG CTG CTG AAG TTA 6891  
 Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu  
 220 225 230 235

TGG AAA CAT CAA AAC AAA GAC CAA GAT ATA GTC AAG AAG ATC ATC CAA G 6940  
 Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile Ile Gln  
 240 245 250

GTATGATAAT CTAAAATAAA AAGATCAATC AGAAATCAAA GACACCTATT TATCATAAAC 7000  
 CAGGAACAAG ACTGCATGTA TGTTTAGTTG TGTGGATCTT GTTCCCTGT TGGAATCATT 7060  
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 TTTTAATGGC ATATGTTATG AGAATTAATG AGATAAAATC TGAAAAGTGT TTGAGCCTCT 7480  
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 CTCCTTTAGA AATGGTGTGA GAGAGAAACA GAGAGAGATA GGGAGAGAAG TGTGAAAGAA 7600  
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 AGTTCTGACT TCAGTAAGCA TTGGGAGGAC ATGCTAGAAG AAAAAGGAAG AAGAGTTTCC 7720  
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 TATAGTCTTG CACTACCCTA AAAAATTCA AGTATCTGAA ACCGGGGCAA CAGATTTTAG 7840  
 GAGACCAACG TCTTTGAGAG CTGATTGCTT TTGCTTATGC AAAGAGTAAA CTTTTATGTT 7900  
 TTGAGCAAAC CAAAAGTATT CTTTGAACGT ATAATTAGCC CTGAAGCCGA AAGAAAAGAG 7960  
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 TTCTCTAGGC TGTGTGTCTC CTTTAGTTCC TCGACCTCAT GTCTTTGAGT TTTCAGATAT 8200  
 CCTCCTCATG GAGGTAGTCC TCTGGTGCTA TGTGTATTCT TTAAAGGCTA GTTACGGCAA 8260  
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Asp Ile Asp

CTC TGT GAA AAC AGC GTG CAG CGG CAC ATT GGA CAT GCT AAC CTC ACC 8724  
 Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala Asn Leu Thr  
 255 260 265 270

TTC GAG CAG CTT CGT AGC TTG ATG GAA AGC TTA CCG GGA AAG AAA GTG 8772  
 Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly Lys Lys Val  
 275 280 285

GGA GCA GAA GAC ATT GAA AAA ACA ATA AAG GCA TGC AAA CCC AGT GAC 8820  
 Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys Pro Ser Asp  
 290 295 300

CAG ATC CTG AAG CTG CTC AGT TTG TGG CGA ATA AAA AAT GGC GAC CAA 8868

Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn Gly Asp Gln

305

310

315

GAC ACC TTG AAG GGC CTA ATG CAC GCA CTA AAG CAC TCA AAG ACG TAC 8916

Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser Lys Thr Tyr

320

325

330

CAC TTT CCC AAA ACT GTC ACT CAG AGT CTA AAG AAG ACC ATC AGG TTC 8964

His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe

335

340

345

350

CTT CAC AGC TTC ACA ATG TAC AAA TTG TAT CAG AAG TTA TTT TTA GAA 9012

Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu

355

360

365

ATG ATA GGT AAC CAG GTC CAA TCA GTA AAA ATA AGC TGC TTA 9054

Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu

370

375

380

TAACTGGAAA TGGCCATTGA GCTGTTTCCT CACAATTGGC GAGATCCCAT GGATGAGTAA 9114

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CCTTACTAAA TATGGGAATG TCTAACTTAA ATAGCTTTGG GATTCCAGCT ATGCTAGAGG 9414

CTTTTATTAG AAAGCCATAT TTTTCTCTGT AAAAGTTACT AATATATCTG TAACACTATT 9474

ACAGTATTGC TATTTATATT CATTCAGATA TAAGATTGG ACATATTATC ATCCTATAAA 9534  
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 ATATTAGATG CTCTGAGAAA TTGAATGTAC CTTATTTAAA AGATTTTATG GTTTTATAAC 9834  
 TATATAAATG ACATTATTAA AGTTTTCAAA TTATTTTTTA TTGCTTTCTC TGTTGCTTTT 9894  
 ATTT 9898

Sequence number: 3

Length of sequence: 401

Sequence Type: amino acid

Strandedness: single stranded

Topology: linear

Molecular type: protein

Sequence:

Met	Asn	Asn	Leu	Leu	Cys	Cys	Ala	Leu	Val	Phe	Leu	Asp	Ile	Ser
	-20				-15					-10				
Ile	Lys	Trp	Thr	Thr	Gln	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His
	-5				1					5				
Tyr	Asp	Glu	Glu	Thr	Ser	His	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro
10					15					20				
Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	Cys	Thr	Ala	Lys	Trp	Lys	Thr
25					30					35				
Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	Tyr	Thr	Asp	Ser	Trp	His
40					45					50				

Ile Gln Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile		
250	255	260
Gly His Ala Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu		
265	270	275
Ser Leu Pro Gly Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr		
280	285	290
Ile Lys Ala Cys Lys Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser		
295	300	305
Leu Trp Arg Ile Lys Asn Gly Asp Gln Asp Thr Leu Lys Gly Leu		
310	315	320
Met His Ala Leu Lys His Ser Lys Thr Tyr His Phe Pro Lys Thr		
325	330	335
Val Thr Gln Ser Leu Lys Lys Thr Ile Arg Phe Leu His Ser Phe		
340	345	350
Thr Met Tyr Lys Leu Tyr Gln Lys Leu Phe Leu Glu Met Ile Gly		
355	360	365
Asn Gln Val Gln Ser Val Lys Ile Ser Cys Leu		
370	375	380

Sequence number: 4

Length of sequence: 1206

Sequence Type: nucleic acid

Strandedness: single stranded

Topology: linear

Molecular type: cDNA

Sequence:

ATGAACAACCT TGCTGTGCTG CGCGCTCGTG TTTCTGGACA TCTCCATTAA GTGGACCACC 60  
 CAGGAAACGT TTCCTCCAAA GTACCTTCAT TATGACGAAG AAACCTCTCA TCAGCTGTTG 120  
 TGTGACAAAT GTCCTCCTGG TACCTACCTA AAACAACACT GTACAGCAAA GTGGAAGACC 180  
 GTGTGCGCCC CTTGCCCTGA CCACTACTAC ACAGACAGCT GGCACACCAG TGACGAGTGT 240  
 CTATACTGCA GCGCCGTGTG CAAGGAGCTG CAGTACGTCA AGCAGGAGTG CAATCGCACC 300  
 CACAACCGCG TGTGCGAATG CAAGGAAGGG CGCTACCTTG AGATAGAGTT CTGCTTGAAA 360  
 CATAGGAGCT GCCCTCCTGG ATTTGGAGTG GTGCAAGCTG GAACCCCAAG GCGAAATACA 420  
 GTTTGCAAAA GATGTCCAGA TGGGTTCTTC TCAAATGAGA CGTCATCTAA AGCACCCCTGT 480  
 AGAAAACACA CAAATTGCAG TGTCTTTGGT CTCCTGCTAA CTCAGAAAGG AAATGCAACA 540  
 CACGACAACA TATGTTCCGG AAACAGTGAA TCAACTCAAA AATGTGGAAT AGATGTTACC 600  
 CTGTGTGAGG AGGCATTCTT CAGGTTTGCT GTTCCTACAA AGTTTACGCC TAACTGGCTT 660  
 AGTGTCTTGG TAGACAATTT GCCTGGCACC AAAGTAAACG CAGAGAGTGT AGAGAGGATA 720  
 AAACGGCAAC ACAGCTCACA AGAACAGACT TTCCAGCTGC TGAAGTTATG GAAACATCAA 780  
 AACAAAGACC AAGATATAGT CAAGAAGATC ATCCAAGATA TTGACCTCTG TGAAAACAGC 840  
 GTGCAGCGGC ACATTGGACA TGCTAACCTC ACCTTCGAGC AGCTTCGTAG CTTGATGGAA 900  
 AGCTTACCGG GAAAGAAAGT GGGAGCAGAA GACATTGAAA AAACAATAAA GGCATGCAAA 960  
 CCCAGTGACC AGATCCTGAA GCTGCTCAGT TTGTGGCGAA TAAAAAATGG CGACCAAGAC 1020  
 ACCTTGAAGG GCCTAATGCA CGCACTAAAG CACTCAAAGA CGTACCACTT TCCCAAAACT 1080  
 GTCATCAGA GTCTAAAGAA GACCATCAGG TTCCTTCACA GCTTCACAAT GTACAAATTG 1140  
 TATCAGAAAGT TATTTTLAGA AATGATAGGT AACCAGGTCC AATCAGTAAA AATAAGCTGC 1200  
 TTATAA 1206



CLAIMS:

1. A DNA comprising the nucleotide sequences of the Sequences No. 1 and No. 2 in the Sequence Table.

2. The DNA according to claim 1, wherein the Sequence ID No. 1 includes the first exon of the OCIF gene and the Sequence ID No. 2 includes the second, third, fourth, and fifth exons.

3. A protein exhibiting the activity of inhibiting differentiation and/or maturation of osteoclasts and having the following physicochemical characteristics,

(a) molecular weight (SDS-PAGE):

(i) Under reducing conditions: about 60 kD,

(ii) Under non-reducing conditions: about 60 kD and about 120 kD;

(b) amino acid sequence:

includes an amino acid sequence of the Sequence ID No. 3 in the Sequence Table,

(c) affinity:

exhibits affinity to a cation exchanger and heparin, and

(d) heat stability:

(i) the osteoclastogenesis-inhibitory activity is reduced when treated with heat at 70°C for 10 minutes or at 56°C for 30 minutes,

(ii) the osteoclastogenesis-inhibitory activity is lost when treated with heat at 90°C for 10 minutes.

4. A process for producing a protein exhibiting an

activity of inhibiting differentiation and/or maturation of osteoclasts and having the following physicochemical characteristics,

(a) molecular weight (SDS-PAGE):

(i) Under reducing conditions: about 60 kD,

(ii) Under non-reducing conditions: about 60 kD and about 120 kD;

(b) amino acid sequence:

includes an amino acid sequence of the Sequence ID No. 3 of the Sequence Table,

(c) affinity:

exhibits affinity to a cation exchanger and heparin, and

(d) heat stability:

(i) the osteoclastogenesis-inhibitory activity is reduced when treated with heat at 70°C for 10 minutes or at 56°C for 30 minutes,

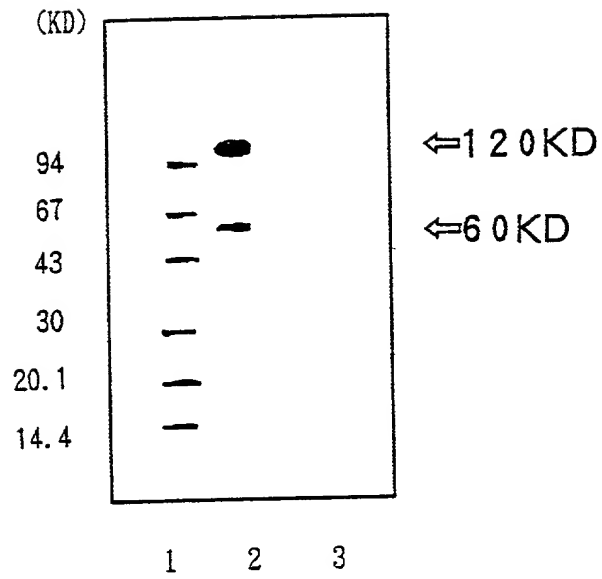
(ii) the osteoclastogenesis-inhibitory activity is lost when treated with heat at 90°C for 10 minutes,

the process comprising inserting a DNA including the nucleotide sequences of the sequences No. 1 and No. 2 in the Sequence Table into an expression vector, producing a vector capable of expressing a protein having the above-mentioned physicochemical characteristics and exhibiting the activity of inhibiting differentiation and/or maturation of osteoclasts, and producing this protein by a genetic engineering technique.

ABSTRACT OF THE DISCLOSURE

DNAs having the nucleotide sequences of the Sequences No. 1 and No. 2 in the Sequence Table and a process for producing a protein which comprises inserting these DNAs into expression vectors to thereby produce a protein having molecular weights of about 60 kD (under reductive conditions) and about 60 kD and 120 kD (under non-reductive conditions) and being capable of inhibiting formation of osteoclast. These proteins are useful in the treatment of osteoporosis and rheumatism.

Figure 1



## PATENT

Atty. Docket No. FJN-063  
(3999/66)**COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION**

(Original, Design, National Stage of PCT, Supplemental, Divisional, Continuation or CIP)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**NOVEL DNA AND PROCESS FOR PREPARING PROTEIN USING THE DNA**

the specification of which (check one):

is attached hereto.

☒ was filed on April 16, 1998 as Application Serial No. 09/051,670 or

was described and claimed in PCT International Application No. filed on and as amended under PCT Article 19 on (if any).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to herein.

I acknowledge the continuing duty to disclose information which is material to the examination of this application in accordance with 37 C.F.R. §1.56.

**PRIORITY CLAIM**

- A. I hereby claim benefit under 35 U.S.C. 119(e) of United States Provisional Application No. , filed on .
- B. I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and I have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.  
no such applications have been filed.

Country	Application Number	Date of Filing (mo., day, year)	Priority Claimed Under 35 USC 119	
			YES	NO
			YES	NO
			YES	NO

Country	Application Number	Date of Filing (mo., day, year)	Priority Claimed Under 37 USC 119
Japan	235928/1996	August 19, 1996	YES

- PRIOR U.S. NON-PROVISIONAL APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC §120:**

U.S. APPLICATIONS	U.S. FILING DATE	STATUS
PCT/JP97/02859	August 19, 1997	Pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)

a) 1997-1998		b) 1998-1999		c) 1999-2000		d) 2000-2001		e) 2001-2002		f) 2002-2003		g) 2003-2004		h) 2004-2005		i) 2005-2006		j) 2006-2007		k) 2007-2008		l) 2008-2009		m) 2009-2010		n) 2010-2011		o) 2011-2012		p) 2012-2013		q) 2013-2014		r) 2014-2015		s) 2015-2016		t) 2016-2017		u) 2017-2018		v) 2018-2019		w) 2019-2020		x) 2020-2021		y) 2021-2022		z) 2022-2023		aa) 2023-2024		ab) 2024-2025		ac) 2025-2026		ad) 2026-2027		ae) 2027-2028		af) 2028-2029		ag) 2029-2030		ah) 2030-2031		ai) 2031-2032		aj) 2032-2033		ak) 2033-2034		al) 2034-2035		am) 2035-2036		an) 2036-2037		ao) 2037-2038		ap) 2038-2039		aq) 2039-2040		ar) 2040-2041		as) 2041-2042		at) 2042-2043		au) 2043-2044		av) 2044-2045		aw) 2045-2046		ax) 2046-2047		ay) 2047-2048		az) 2048-2049		ba) 2049-2050		bb) 2050-2051		bc) 2051-2052		bd) 2052-2053		be) 2053-2054		bf) 2054-2055		bg) 2055-2056		bh) 2056-2057		bi) 2057-2058		bj) 2058-2059		bk) 2059-2060		bl) 2060-2061		bm) 2061-2062		bn) 2062-2063		bo) 2063-2064		bp) 2064-2065		bq) 2065-2066		br) 2066-2067		bs) 2067-2068		bt) 2068-2069		bu) 2069-2070		bv) 2070-2071		bw) 2071-2072		bx) 2072-2073		by) 2073-2074		bz) 2074-2075		ca) 2075-2076		cb) 2076-2077		cc) 2077-2078		cd) 2078-2079		ce) 2079-2080		cf) 2080-2081		cg) 2081-2082		ch) 2082-2083		ci) 2083-2084		cj) 2084-2085		ck) 2085-2086		cl) 2086-2087		cm) 2087-2088		cn) 2088-2089		co) 2089-2090		cp) 2090-2091		cq) 2091-2092		cr) 2092-2093		cs) 2093-2094		ct) 2094-2095		cu) 2095-2096		cv) 2096-2097		cw) 2097-2098		cx) 2098-2099		cy) 2099-2100		cz) 2100-2101		da) 2101-2102		db) 2102-2103		dc) 2103-2104		dd) 2104-2105		de) 2105-2106		df) 2106-2107		dg) 2107-2108		dh) 2108-2109		di) 2109-2110		dj) 2110-2111		dk) 2111-2112		dl) 2112-2113		dm) 2113-2114		dn) 2114-2115		do) 2115-2116		dp) 2116-2117		dq) 2117-2118		dr) 2118-2119		ds) 2119-2120		dt) 2120-2121		du) 2121-2122		dv) 2122-2123		dw) 2123-2124		dx) 2124-2125		dy) 2125-2126		dz) 2126-2127		ea) 2127-2128		eb) 2128-2129		ec) 2129-2130		ed) 2130-2131		ee) 2131-2132		ef) 2132-2133		eg) 2133-2134		eh) 2134-2135		ei) 2135-2136		ej) 2136-2137		ek) 2137-2138		el) 2138-2139		em) 2139-2140		en) 2140-2141		eo) 2141-2142		ep) 2142-2143		eq) 2143-2144		er) 2144-2145		es) 2145-2146		et) 2146-2147		eu) 2147-2148		ev) 2148-2149		ew) 2149-2150		ex) 2150-2151		ey) 2151-2152		ez) 2152-2153		fa) 2153-2154		fb) 2154-2155		fc) 2155-2156		fd) 2156-2157		fe) 2157-2158		ff) 2158-2159		fg) 2159-2160		fh) 2160-2161		fi) 2161-2162		fj) 2162-2163		fk) 2163-2164		fl) 2164-2165		fm) 2165-2166		fn) 2166-2167		fo) 2167-2168		fp) 2168-2169		fq) 2169-2170		fr) 2170-2171		fs) 2171-2172		ft) 2172-2173		fu) 2173-2174		fv) 2174-2175		fw) 2175-2176		fx) 2176-2177		fy) 2177-2178		fz) 2178-2179		ga) 2179-2180		gb) 2180-2181		gc) 2181-2182		gd) 2182-2183		ge) 2183-2184		gf) 2184-2185		gg) 2185-2186		gh) 2186-2187		gi) 2187-2188		gj) 2188-2189		gk) 2189-2190		gl) 2190-2191		gm	
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Reg. No. 41,418

Patent Administrator  
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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